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Oral delivery of non-viral nucleic acid-based therapeutics - *do we have the guts for this?*

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ABSTRACT

Gene therapy with RNA and pDNA-based drugs is limited by poor enzymatic stability and poor cellular permeation. The delivery of nucleic acids, in particular by the oral route, remains a major hurdle. This review will focus on the barriers to the oral delivery of nucleic acids and the strategies, in particular formulation strategies, which have been developed to overcome these barriers. Due to their very low oral bioavailability, the most obvious and most investigated biomedical applications for their oral delivery are related to the local treatment of inflammatory bowel diseases and colorectal cancers. Preclinical data but not yet clinical studies support the potential use of the oral route for the local delivery of formulated nucleic acid-based drugs.

1. Introduction

The improved understanding of the genetic roots of numerous diseases going hand in hand with the completion of the human genome project opened the door for the discovery of novel therapeutics specifically modulating the expression of disease-relevant genes. Generally, these therapeutics can be categorized into viral and non-viral formulations. The non-viral ones offering the prospect of avoiding oncogenic risk and of managing potentially larger payloads are either plasmid DNA (pDNA) encoding for therapeutic proteins such as GLP-1 (Nurunnabi et al., 2017) or insulin (Rothman et al., 2005) and in case of DNA vaccines for antigens (Sinha et al., 2017) or RNA-based drugs including antisense oligonucleotides, short interfering RNA (siRNA; Vaidya et al., 2019; Jain et al., 2017; Zhao et al., 2018), microRNA (miRNA; Brown et al., 2018), messenger RNA (mRNA; Sago et al., 2018; McKinlay et al., 2018), and Aptamer (Z. Chen et al., 2017). In contrast to all other drugs they can edit genes curing consequently genetic defects (Shim et al., 2017). Furthermore, they can be used to shut off certain gene expression. In the case of certain gastrointestinal diseases, such as inflammatory bowel disease (IBD) and colon cancer, where current drug therapies are inadequate such gene-based therapeutics may have significant clinical benefits.

Despite these convincing advantages, however, progress made over the last decades towards a clinical use of nucleic acid-based therapeutics is quite minor as in particular the delivery of these drugs still remains a major hurdle. As the gastrointestinal tract (GIT) is likely the most hostile environment in the entire body for nucleic acid-based therapeutics it may seem brazen at first sight to focus on their oral administration. Within this review, however, we want to convince our audience that despite all the barriers associated with the GIT the oral administration of nucleic acid-based drugs is not a 'mission impossible' and quite the contrary that this route bears even advantages and opportunities over others.

2. Physicochemical considerations

Therapeutic nucleic acids which have been investigated in recent years include plasmid DNA, RNA therapies such as siRNA, microRNA, shRNA, mRNA and gene editing tools such as CRISPR. Nucleic acids (NA) all have similar chemical structure, but differences arise in terms of size, stability and mechanisms/site of action. These molecules, versus traditional small drug molecules, have relatively large molecular weights (siRNA 21–23 nucleotides, pDNA several kilobase pairs). They are hydrophilic and negatively charged due to the phosphate backbone. As a result of these physicochemical properties membrane permeability

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is low. In addition, the charge and size may affect diffusion through mucus as found on the absorptive surface of the gut. These molecules are also prone to enzymatic degradation and plasma half-lives of < 1 min (Godinho et al., 2014) have been reported. Pre-incubation of a cyclodextrin/DNA complex in simulated intestinal fluid (SIF) containing pancreatin, for instance, significantly decreased the transfection efficacy in Caco-2 cells (O'Neill et al., 2013).

To help overcome the limitations to delivery imposed by these physicochemical properties structural modifications have been explored. Two approaches which have been particularly successful in the case of siRNA involve chemical conjugation of siRNA to bioactive entities, and secondly the synthesis of hydrophobic siRNA derivatives.

The advantages associated with conjugates include a defined chemical structure, and the potential for a simpler formulation approach which may be easier to manufacture and scale-up (Kaczmarek et al., 2017). Direct conjugation of siRNA to a bioactive targeting ligand e.g. GalNac for targeting hepatocytes, (www.alnylam.com) facilitates cellular uptake and can alter the biodistribution of the nucleic acids by ensuring cell/site specific delivery. Conjugation to lipophiles, e.g. cholesterol, can also enhance membrane permeability relative to the unmodified NA (Gooding et al., 2016; Malhotra et al., 2018; Wong et al., 2012). PEGylation of siRNA can lead to prolonged plasma exposure and gene silencing efficiency (Godinho et al., 2014), PEG conjugates may also be useful in the gut as a stearic barrier to enzymatic degradation.

The stability, efficacy and biodistribution of hydrophobically modified siRNA (hsiRNA) has also been extensively investigated. Alterman et al. (2015) explained that "hsiRNA is an asymmetric compound composed of a 15-nucleotide modified RNA duplex with a single-stranded 3′ extension on the guide strand. Pyrimidines in the hsiRNA are modified with 2′-O-methyl (passenger strand) or 2′-fluoro (guide strand) to enhance stability, and the 3′ end of the passenger strand is conjugated to a hydrophobic teg-Chol (tetraethylene glycol cholesterol) to promote membrane binding. The single-stranded tail contains hydrophobic phosphorothioate linkages thus promoting cellular uptake. The inclusion of phosphorothioates, ribose modifications, and a cholesterol conjugate contribute to overall hydrophobicity and are reported to be necessary for stability and for effective cellular uptake". These hsiRNA are non-immunogenic and have produced gene silencing in an in vivo mouse model of Huntington's disease without any additional formulation (Didiot et al., 2016).

While the success of these approaches has to date been assessed following parenteral administration, potential exists to explore these technologies for oral or rectal delivery of therapeutic nucleic acids.

3. State of the art

3.1. Formulation strategies

Fig. 1 presents the various state-of-the-art drug delivery systems evaluated for the oral administration of nucleic acid-based therapeutics. These systems were classified in four classes: lipid-based systems, e.g. liposomes, lipoplexes, self-emulsifying drug delivery systems (SEDDS); polymer-based systems, e.g. chitosan, trimethylchitosan, polyethylenimine (PEI), Poly Lactic-co-Glycolic Acid (PLGA); surface decoration, e.g. with polyethylene glycol (PEG), antibody, galactose, mannose; and finally, hybrid systems, e.g. nanogel, gold-PEI particles, and Nanoparticles-in-microspheres (NiMOS).

3.1.1. Enzymatic barrier and strategies to overcome it

Having reached the intestinal fluid nucleic acid-based therapeutics face an enzymatic barrier of nucleases. In contrast to most other gastrointestinal (GI)-enzymes such as proteases, where all types of enzymes, their activity and even recognition sites on peptides and proteins are known (Bernkop-Schnürch, 1998), almost nothing is known about GI-nucleases. At least we know from various studies, that nucleic acid-based drugs are extensively degraded by GI-nucleases. Already in the 1970s it was shown that plasmid DNA is rapidly degraded when added to just low dilutions of rat intestinal contents. This observation let the authors of this study to conclude that the transmission of naked recombinant DNA in the intestinal tract is highly improbable (Maturin and Curtiss, 1977). Results are in agreement with those of Loretz et al. showing almost entire degradation of plasmid DNA in freshly withdrawn and diluted porcine gastric and intestinal fluid within an hour. The nuclease activity in the intestinal fluid was thereby determined to correspond to 20 Kunitz mUnits of DNAse I/ml (Loretz et al., 2006). In another study the enzymatic degradation of different antisense oligonucleotides in a fasted rat small intestine homogenate model was investigated. The tested phosphodiester oligonucleotide was completely degraded within 30 min, whereas just half of a phosphorothioate oligonucleotide was metabolized within 4 h demonstrating that depending on the type of modification more or less nuclease stable nucleic acid-based therapeutics can be designed (González Ferreiro et al., 2003).

To avoid degradation by nucleases orally administered nucleic acid-based drugs are either chemically modified in order to make them more resistant towards enzymes or incorporated in nanocarrier systems shielding against enzymatic attack. Broadly speaking, chemical modifications of nucleic acids are either based on internucleotide linkage (I), nucleobase (II) or sugar modifications (III). As internucleotide phosphodiester linkages of DNA and RNA are the target of cleavage by *endo-* and exonucleases, internucleotide linkage modifications including phosphorothioates, N3' phosphoramidates, boranophosphate 2′,5′–phosphodiesters, amide-linked phosphonoacetates, morpholinos and peptide nucleic acids are mainly used in order to improve stability (Deleavey and Damha, 2012). This strategy is certainly first choice for synthesized nucleic acid-based therapeutics as the effort of synthesizing a DNA- or RNA-sequence utilizing unmodified or modified nucleotides is more or less the same. Moreover, as synthesized nucleic acid-based drugs are comparatively small and, in most cases, supposed to reach the systemic circulation, chemical modifications are also helpful to reduce the systemic metabolism and consequently to prolong elimination half-life. For nucleic acid-based therapeutics that cannot be sufficiently stabilized by modifications or that are simply too big to be chemically modified such as plasmids, however, this strategy does not lead to the desired result. For this type of nucleic acid-based drugs protection towards nucleases can only be achieved by carrier systems shielding on the one hand the incorporated drug towards nucleases and delivering on the other hand their payload at least to the apical membrane of enterocytes or even beyond where the drug cannot be degraded anymore by nucleases of the intestinal fluid. In order to avoid free access of nucleases to nucleic acid-based therapeutics they can be co-precipitated with cationic polymers such as polyethyleneimine (Banerjee et al., 2006), chitosan (Ragelle et al., 2014), thiolated chitosans (Zhang et al., 2013) or dendrimers (Bielinska et al., 1997). Utilizing this technique, nanoparticles can be simply generated providing at least to a certain extent a protective effect. Alternatively, nucleic acid-based therapeutics can be complexed with cationic surfactants such as DOPA or DOTA also forming nanoparticles. The protective effect towards nucleases, however, seems to be considerably low. Hauptstein et al. (2015), for instance, showed a rapid degradation of pDNA although complexed with Lipofectin. Utilizing liposomes is another approach. Because of their hydrophilic character, nucleic acid-based drugs assemble not just on the inner phospholipid bilayers, where they maybe protected, but also on the outer bilayer where they are not at all protected from nucleases. Due to the addition of cationic lipids, however, nucleic acid-based drugs are coated by a lipophilic shell likely assembling in lipophilic regions of liposomes where they are to a high extend protected towards nucleases (Cortesi et al., 1996). A potentially efficient strategy to provide protection towards nucleases is the use of SEDDS. Due to a hydrophobic ion pairing (HIP) with cationic surfactants nucleic acid-based drugs can be efficiently incorporated in SEDDS pre-concentrates. As nucleases are very hydrophilic, they cannot penetrate into the oily droplets formed in the intestinal fluid. A comparision of the protective effect of cationic lipid complexes with that of SEDDS as illustrated in Fig. 2 demonstrates the potential of this strategy (Hauptstein et al., 2015).

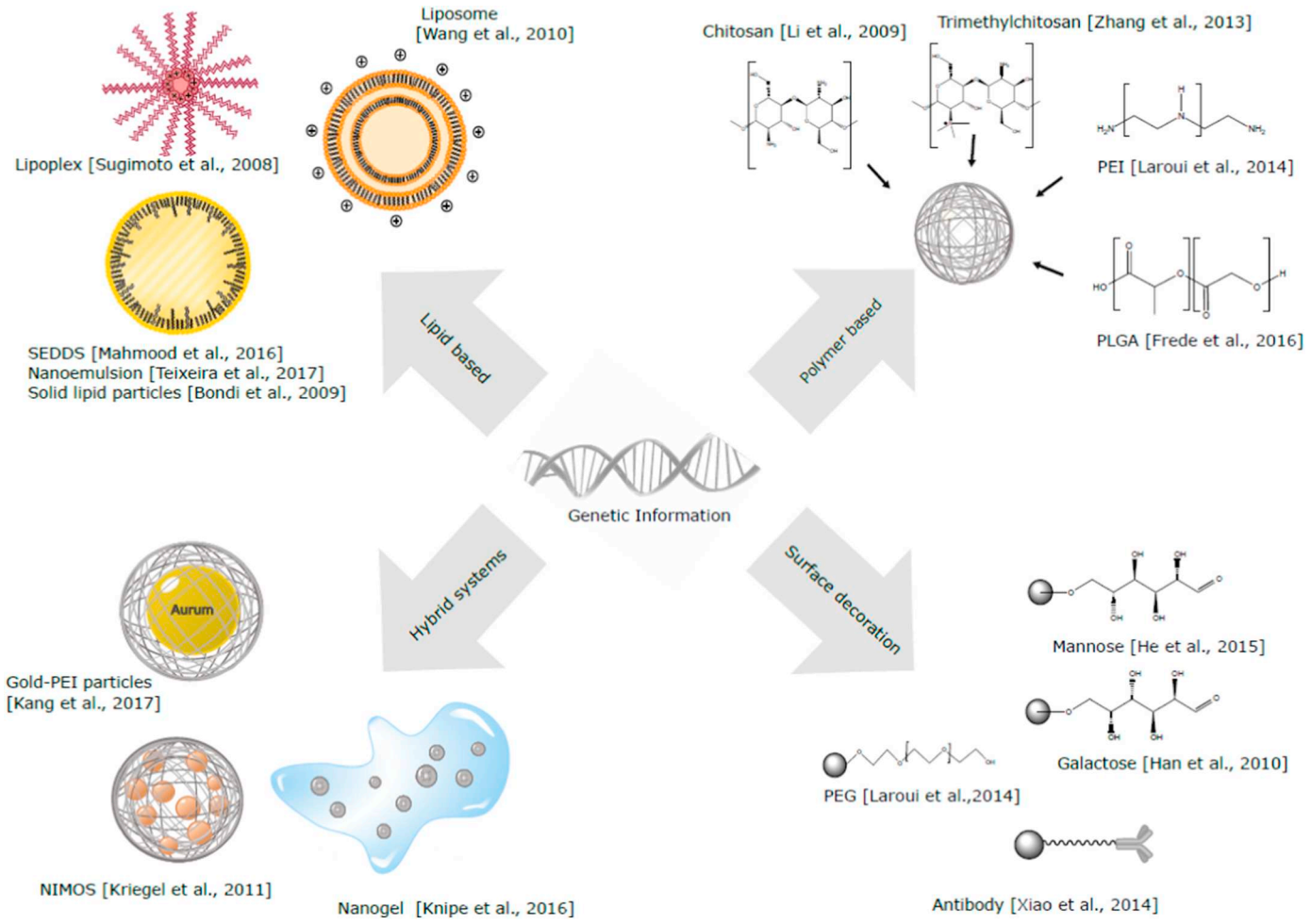

Fig. 1. State-of-the-art drug delivery systems for oral administration of nucleic acid-based therapeutics.

Another strategy is making use of nuclease inhibitors that are co-administered with the nucleic acid-based therapeutic in order to suppress the activity of these enzymes during the drug liberation and absorption process. So far, however, very little is known about potent inhibitors of intestinal nucleases. At least EDTA, sodium dodecyl sulfate and aurintricarboxylic acid were proven to exhibit a pronounced inhibitory effect (Loretz et al., 2006). In order to avoid a rapid dilution and absorption of these inhibitors in the intestine leaving the comparatively poorly absorbed therapeutic agent unprotected behind in the intestinal lumen, these inhibitors can be immobilized on not absorbable polymers functioning as drug carrier matrix. An example for such systems is chitosan-EDTA conjugate (Loretz et al., 2006). If just a very few drug administrations are needed to achieve the therapeutic aim such as in case of DNA vaccines, the strategy seems promising. In case of long-term treatments, however, side effects will likely be a hindrance. At least we know from orally administered protease inhibitors that the body compensates the suppressed enzymatic activity in the intestine via a feedback regulation loop resulting not just in an increased secretion of degrading enzymes but for instance in case of serine proteases also in a hypertrophy and hyperplasia of the pancreas e.g. (Otsuki et al., 1987). Taken all, whenever chemical modifications on therapeutic nucleic acids are feasible in order to provide stability towards nucleases, it is preferred over all other strategies. Unless, nanocarrier systems are likely the most promising alternative.

3.1.2. Mucus barrier and strategies to overcome it

Having overcome this enzymatic barrier nucleic acid-based therapeutics are confronted with another huge barrier namely the mucus gel layer. Mucus glycoproteins designated mucins exhibit cysteine-rich subdomains that are crosslinked with each other via disulphide bonds. They built up a three-dimensional network with a mesh size of ~10–200 nm within its microstructure that blocks most pathogens and xenobiotics too large to permeate it. In contrast to the microstructure of mucus with a defined mesh size, its macrostructure is more heterogeneous. Because of this heterogenicity and considering that the mucus is not a static but a very dynamic system, the network is even for an invader larger than 200 nm to some extent leaky. Furthermore, the region of Payer's patches is less covered by a mucus layer. This slight leakiness of the mucus layer, however, is in most cases insufficient for an efficient nucleic acid-based drug delivery, apart from this size dependent barrier large hydrophilic molecules are trapped in the mucus by its adhesive nature. Due to the formation of numerous non-covalent bonds such as ionic interactions, hydrogen bonds and van-der-Waals forces they are immobilized within the mucus (Bernkop-Schnürch and Fragner, 1996). Taking its adhesive nature, the thickness of the mucus layer in the small intestine of up to 400 µm and an estimated mucus turnover in the range of 1–4.5 h (Lehr et al., 1991) into account, the mucus layer is for small nucleic acid-based therapeutics at least a hindrance and for pDNA it is an almost impermeable barrier. As in case of an oral gene therapy of gut related diseases such as cystic fibrosis (Ooi and Durie, 2016) or lactose intolerance (During et al., 1998) intestinal stem cells being in the crypts of Lieberkühn have to be reached, the mucus barrier becomes even more challenging. Although there is a constant water flow towards the intestinal epithelium accelerating the diffusion process of drugs through the mucus gel layer (Fabiano et al., 2017), it is obvious that without the aid of mucolytic agents and/or

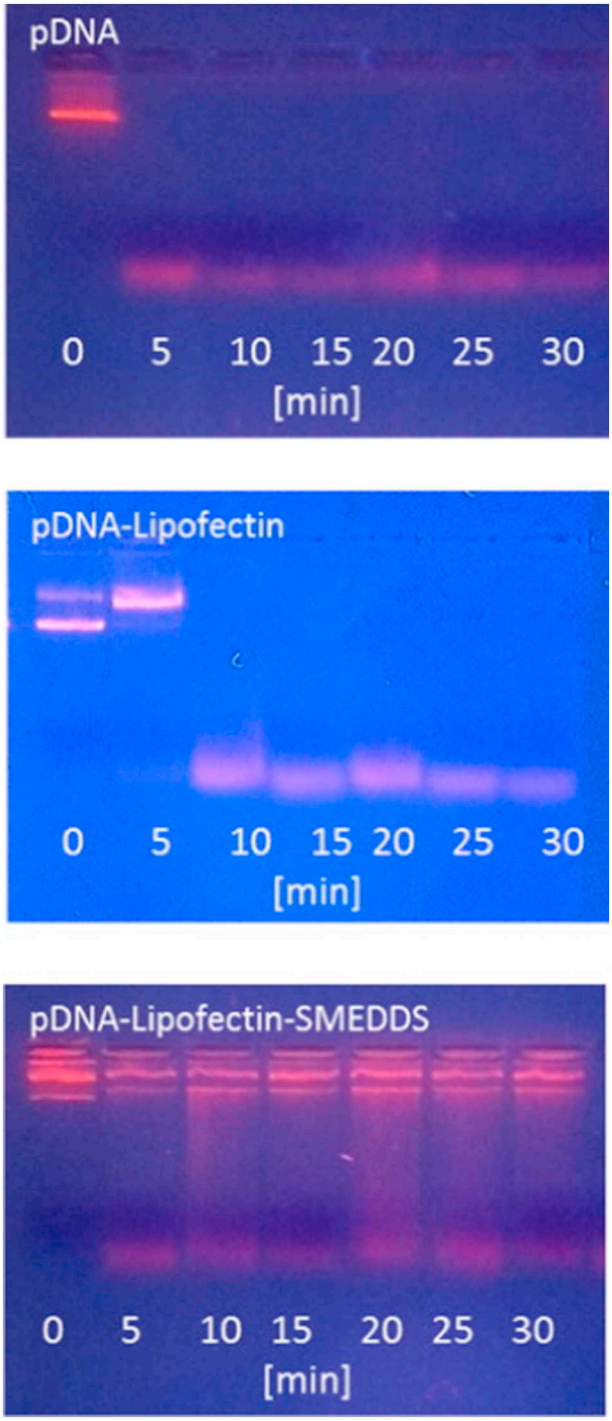

Fig. 2. Results of enzymatic degradation studies with DNase I. Reaction was stopped after predetermined time points and extent of degradation was visualized by agarose gel electrophorese. Gel was prestained with GelRED® and detected under UV light. Gel shows results for a 1% naked pDNA solution, 1% resuspended pDNA–Lipofectin® complex and 1% nanoemulsion of pDNA–Lipopfectin® complex loaded SEDDS.
Adopted from Hauptstein et al. (2015).

appropriate drug carriers' nucleic acid-based therapeutics are losing potential because of the mucus gel barrier.

In order to permeate the mucus gel layer in high quantities the drug delivery system has to be small enough – preferably < 100 nm – and has to be mucoinert. Nanoparticles formed of nucleic acid-based therapeutics and cationic polymers exhibiting in combination a negative zeta potential seem to fulfill these demands. They can be generated with a mean size below 100 nm. Moreover, combining anionic and cationic polymers in these particles results in a high density of anionic and cationic charges on the particle surface making them comparatively slippery. They imitate actually viruses that can easily move across the mucus layer exhibiting also a high density of anionic and cationic charges on their surface (Pereira de Sousa et al., 2015). Nevertheless, such systems as well as liposomes exhibit still lower mucus permeating properties than SEDDS. PEG coatings on nanoparticles have been utilized for overcoming various biological barriers to efficient gene delivery, in particular to promote mucus penetration (Suk et al., 2016). As most SEDDS contain PEGylated surfactants in order to guarantee self-emulsification, their comparatively high mucus permeating properties can be explained by PEG substructures assembling on the surface of the oily droplets making them highly mucoinert (Griesser et al., 2018). Although PEGs are certainly helpful in order to overcome the mucus barrier, they are disadvantageous for the particle uptake by epithelial cells. Ball et al. (2018), for instance, observed a decreased efficacy of orally administered siRNA nanoparticles the more PEG they added to these nanocarriers. Because of this PEG dilemma nanocarriers of high density of anionic and cationic charges on their surface are the preferred strategy in order to provide mucoinert properties.

Other strategies are utilizing mucolytic agents such as sulfhydryl compounds cleaving disulfide bonds within the mucus gel layer. Sandberg et al., for instance, could remove mucus both with dithiothreitol and *N*-acetyl-cysteine between the villi but failed to reach the crypt lumen. In addition, as explained by Sandberg et al. (1994), to enhance mucus release from the crypt lumen, they chose "pilocarpine due to its cholinergic properties and preferential binding to muscarinic receptors on crypt goblet cells. Pilocarpine given intraperitoneally 30 min prior to the mucolytic wash resulted in significant eradication of mucus down into the crypt lumen". Furthermore, mucolytic enzymes such as papain or bromelain cleaving certain protein substructures within the mucus gel layer might be another option (Müller et al., 2013). As such mucolytic enzymes do not cleave nucleic acids at all and they make the absorption membrane to some extent more accessible for drug uptake by cleaving tight junctions (Bock et al., 1998), they are among mucolytic agents first choice. In particular the combination of such enzymes with nanocarriers exhibiting a high density of anionic and cationic charges as described above might be a straight forward approach.

3.1.3. *Membrane barrier and strategies to overcome it*

The epithelium lining of the GI-tract portrays another and the likely most challenging barrier to orally administered nucleic acid-based drugs. Brush border microvilli present a variety of charged molecular species, including polar carbohydrates and charged amino acid side chains, thus providing a high-density negative charge on the microvilli at the enterocytes' apical surface (Bennett et al., 2014). Due to this anionic charge, it is primarily a challenge for nucleic acids to reach and be attached to the cell membrane. Even if they overcome this repulsion effect, the situation does not take a turn for the better. Because of their hydrophilic macromolecular nature, they are unable to permeate the lipophilic phospholipid bilayer of enterocytes. For nucleic acid-based therapeutics exhibiting a molecular mass < 5 kDa the paracellular route of uptake seems to be an option, whereas for drugs above that size there seems to be no escape. The likely most important way to overcome the cell membrane is via endocytosis. Nucleic acid-based therapeutics internalized via endocytosis, however, are delivered via the early endosomes to lysosomes for degradation (Luzio et al., 2007), to the trans-Golgi network for processing (Gu et al., 2001), or recycled back to the membrane (Maxfield and McGraw, 2004). For RNA the cytosol is already the target site, whereas pDNA still has to reach the nucleus. Although antisense oligonucleotides function in both the cytoplasm and nucleus, localization to different subcellular regions can affect their therapeutic potency (Crooke et al., 2017). In case of systemic delivery nucleic acid-based drugs have to permeate also the

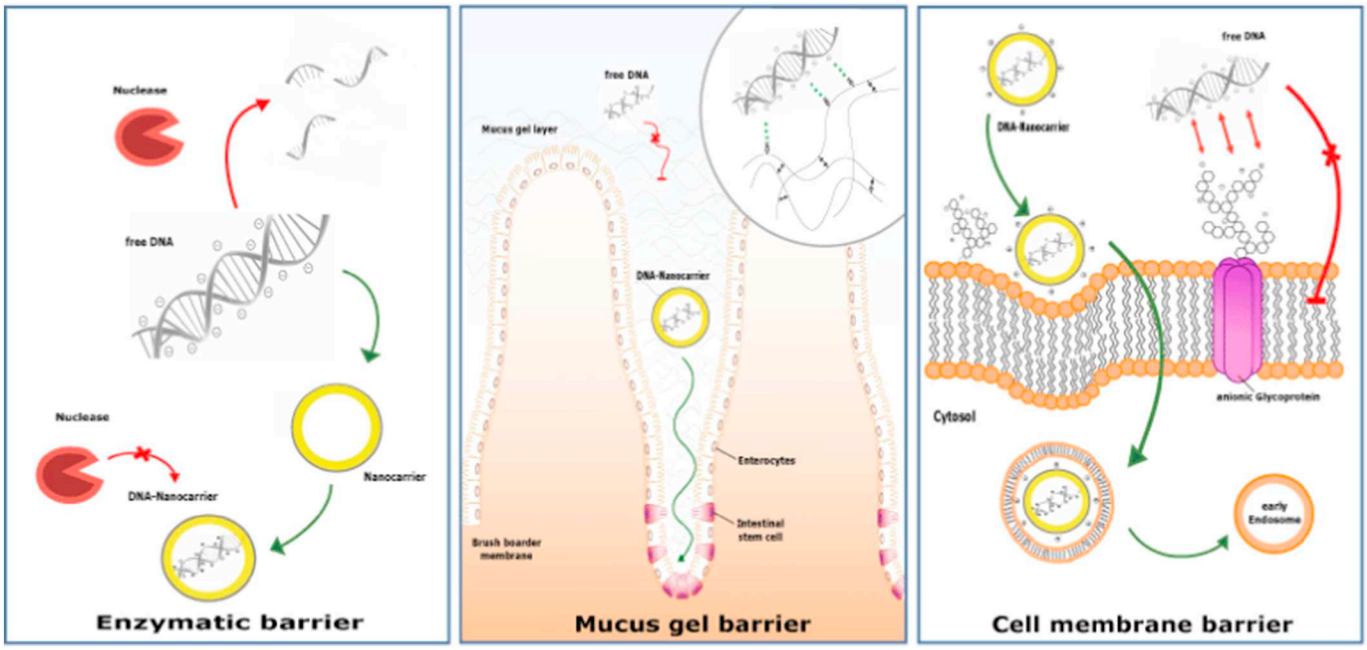

Fig. 3. Physiological barriers in the gastrointestinal tract for nucleic acid-based therapeutics after oral dosing.

plasma membrane in order to reach the systemic circulation. In Fig. 3 the different barriers and routes of uptake are illustrated.

For small nucleic acid-based drugs that are supposed to reach the systemic circulation, permeation enhancers either making the cell membrane leakier or opening up tight junctions have already shown potential in various studies. Raoof et al. (2004), for instance, could achieve an oral bioavailability of 1.4% for an antisense oligonucleotide in dogs when the drug was co-administered with a medium chain fatty acid. Results of this study are illustrated in Fig. 4. An overview about permeation enhancers for oral administration is provided by numerous reviews e.g. (Maher et al., 2016). In order to improve membrane permeability of entire nanocarrier systems, however, mostly other strategies seem to be more efficient. These strategies are mainly focusing on lipophilic (I), cationic (II) and cell penetrating peptide decorated nanocarriers (III).

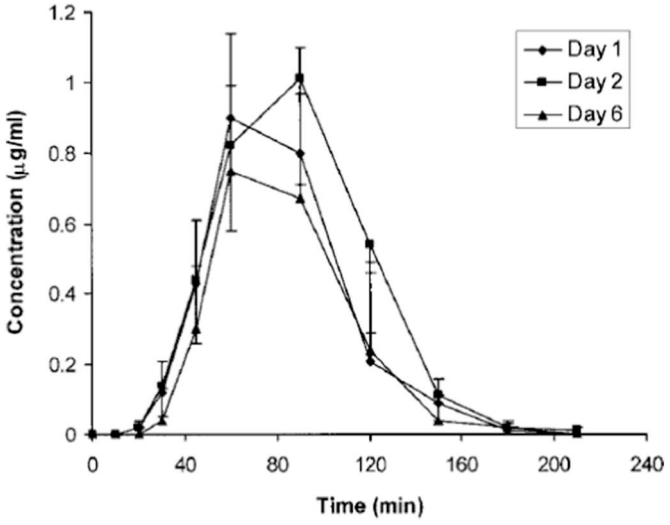

Fig. 4. Mean plasma concentration–time profiles at days 1, 2, and 6 following oral administration of enteric coated tablets containing 80 mg of an antisense oligonucleotide and sodium caprate as permeation enhancer in dogs. Adopted from Raoof et al. (2004).

Since the development of transfection by lipids - designated lipofection (Felgner et al., 1987) - lipoplexes formed between nucleic acid-based therapeutics and cationic lipids are the likely most commonly used strategy to overcome the membrane barrier. Due to the addition of cationic lipids the anionic charges of nucleic acid-based drugs are neutralized and in parallel the lipophilic character is strongly raised. Fusion of these lipophilic complexes with the membrane is consequently facilitated. Cell uptake studies of lipoplexes containing a fluorescent plasmid revealed a very efficient uptake of even 100% and a mechanism that could be different from endocytosis. The escape of the plasmid from intracellular vesicles and the traffic from the cytoplasm to the nucleus, however, were identified as the limiting steps of this approach (Escriou et al., 1998). As lipoplexes are poorly soluble in GI-fluids, the problem of poor membrane permeability seems just to be shifted to a problem of poor solubility. In other words, class 3 drugs are transformed to class 2 drugs without any real improvement in oral bioavailability. According to these considerations lipoplexes cannot be regarded as stand alone strategy. They need to be combined with other strategies providing sufficient solubility and carrying them over the unstirred water layer to the absorption membrane. Lipophilic carrier systems such as SEDDS might fulfill this task. Alternatively, lipophilic nanoparticles can also enter the systemic circulation via the lymphatic route (Murakami et al., 2015).

Cationic nanocarriers are also often used to facilitate nucleic acid-based drug penetration of cells as they interact with the anionic proteoglycans of the cell membrane, depolarize it and induce endocytosis (Poon and Gariépy, 2007; Zhang et al., 2007). The concept seems to work quite efficiently as long as there is no mucus gel layer involved. When these cationic carriers, however, have to permeate the anionic charged mucus layer, they either stick in the mucus or are destabilized due to extensive ionic interactions or they reach the cell membrane having been coated with an anionic layer of mucus neutralizing their cationic charge. Parenterally administered cationic nanocarriers are facing similar problems with anionic serum proteins resulting in the formation of undesirable aggregations or premature release of nucleic acid-based therapeutics. This so-called 'polycation dilemma' (Wang et al., 2012; Bernkop-Schnürch, 2018) has recently been addressed by zeta potential changing carrier systems. Due to an enzymatic cleavage of phosphate substructures from the surface of these drug carrier systems by the membrane bound enzyme intestinal alkaline phosphatase, they change the zeta potential from negative to positive right after

having permeated the mucus gel layer and having reached the membrane (Bonengel et al., 2016).

As most cell penetrating peptides (CPPs) are arginine- and/or lysine-rich peptides exhibiting a cationic charge their effect is often a combination of intrinsic cell penetrating properties and their cationic character. Recently, Mahmood et al. could show an almost 3-fold improved cellular uptake of SEDDS due to the addition of a HIV-1 Tat peptide-oleoyl conjugate. The transfection efficacy of this system was almost twice as high as that achieved with lipofectin (Mahmood et al., 2016). In another study, for instance, peptide vectors containing a laminin receptor-targeting sequence were utilized showing an even 7 times higher transfection efficacy than that of Lipo 2000 (Meng et al., 2018). Although these comparisons with other systems have to be seen with caution, CPPs seem to have potential for oral nucleic acid-based drug delivery. The technology, however, is certainly limited by the degradation of CPPs by proteases and peptidases in the GI-tract. This degradation by gastrointestinal peptidases, however, can be avoided by the use of D-configured instead of L-configured amino acids, as CPPs consisting of D-configured amino acids are on the one hand not cleaved anymore by proteolytic enzymes but on the other hand still recognized by their target site on the cell membrane. Utilizing poly-D-arginine instead of poly-L-arginine is good example for this approach.

Furthermore, the fact that CPP cationic charges are neutralized by the negatively charged mucus substantially compromise their internalization capabilities. Recently, this problem was solved by the covalent attachment of anionic phosphoserine substructures on the top of CPPs heading out of nanocarriers providing an anionic charge on the surface. On the cell membrane these anionic phosphoserine substructures are cleaved off by alkaline phosphatase. Although the concept was used for oral insulin delivery showing a 1.9-fold improved systemic uptake of this therapeutic peptide vs. the same formulation without the phosphoserine substructures, the study provides at least evidence for the potential of this strategy for oral hydrophilic macromolecular drug delivery in general (Wu et al., 2018). The combination of complementary strategies is of course helpful for the design of more efficient oral nucleic acid delivery systems.

3.2. Local versus systemic delivery

Local delivery to the intestine can be achieved via endoscopy techniques, by oral administration where passage through the stomach is an additional barrier, or by rectal administration which avoids the hostile environment of the upper small intestine. Local therapeutic effects on the intestine have also been reported following systemic administration (Peer et al., 2008).

Oral delivery of therapeutic nucleic acid-based therapeutics has many advantages versus systemic administration including; convenience for the patient and thus increased compliance; the potential to treat diseases of the GIT including IBD (Crohn's disease and Colitis) and cancer; and the avoidance of challenges which exist in the blood including interactions with plasma proteins, aggregation and opsonisation. However oral delivery is not a trival matter and major challenges both physicochemical and physiological exist (as described in Section 2). While progress has been made through innovative formulation strategies (Section 3.1) to date clinical translation of oral products has not been realised.

The design of site-specific delivery systems which would restrict release of the nucleic acids to the disease location would be advantageous, for example in the case of IBD and colon cancer which occur in particular areas of the gut and which are often patient specific. Several formulation approaches, including the use of pH-dependent and bioadhesive polymers, have already been investigated and widely reviewed for intestinal site-specific drug delivery, some of these strategies could be applied to the delivery of nucleic acid-based therapeutics.

3.2.1. Inflammatory bowel disease

However, if targeting to the disease site is the aim e.g. in IBD then the physiological changes present in the GIT due to the disease, including pH, transit time, mucous production and the microbiome, which can influence the performance of the delivery system must be taken into consideration (Guo et al., 2016; Hua et al., 2015). The pH in the colon of UC patients is reported to be from 2.3 to 5.5 relative to the healthy colon where the pH range is higher, 6.8 to 7.2 (Hua et al., 2015). Negative effects on delivery can result from the symptoms of the disease including diarrhea which may increase transit times. In contrast, disease related mucosal tissue damage may aid uptake by target cells such as enterocytes and macrophages.

In addition, disease-induced changes in the expression of receptors, adhesion molecules and cell surface proteins have also been reported (Guo et al., 2016; Hua et al., 2015). Several receptors overexpressed in IBD have been investigated for drug delivery using ligand targeted NPs.

Active targeting in IBD: Polystyrene nanoparticles tagged with anti-ICAM-1 antibodies were administered orally to wild type mice C57BL/6. The intercellular adhesion molecule (ICAM-1) is expressed on the surface of GI cells and is upregulated in diseases such as IBD (Mane and Muro, 2012). While up to 60% of antibody was degraded receptor mediated endocytosis in duodenal tissue was demonstrated implying that with further formulation development this receptor may be worth pursuing.

Targeting receptors (mannose and galactose-type lectin) on macrophages, known to be involved in gut inflammation, has also been investigated as a means of enhancing oral delivery of siRNA (Table 1). Mannosylated nanoparticles formulated using a cationic polymer and containing TNF alpha siRNA were effectively taken up in vitro by macrophages and inhibited protein expression in colitis tissue ex vivo (Xiao et al., 2013). Oral administration of galactosylated trimethyl chitosan-cysteine nanoparticles loaded with Map4k4 siRNA decreased the severity of inflammation in a DDS colitis mouse model (Zhang et al., 2013).

Nanoparticles made using poly (lactic acid) poly (ethylene glycol) block copolymer (PLA–PEG) containing TNF alpha siRNA and grafted with a Fab′ portion of the F4/80 Ab (Fab′-bearing) were taken up by macrophages and following oral administration of a hydrogel containing the targeted NPs the symptoms of colitis were attenuated (Laroui et al., 2014). Another target for nanoparticles design is CD98, a type II transmembrane protein overexpressed in gut inflammation (Xiao et al., 2014). Nanoparticles tagged with a surface antibody against CD98 containing CD98 siRNA [CD98 antibody (scCD98)-PEG-urocanic acid-modified chitosan (scCD98-PEG-UAC)/PEI (2 kDa)/siCD98] were administered orally embedded in a chitosan/alginate hydrogel. Approximately 24% of macrophages were seen to take up the nanoparticles resulting in a significant decrease in the severity of the colitis.

In addition to the SEDDS described above (Section 3.1), a range of novel formulation approaches has also been explored for intestinal delivery of nucleic acids. NiMOS have been extensively investigated for oral delivery of pDNA/siRNA mainly for the treatment of IBD (Attarwala et al., 2018; Bhavsar and Amiji, 2007; Kriegel and Amiji, 2011). This formulation has shown promising gene transfection and gene silencing in a dextran sulphate induced colitis mouse model resulting in alleviation of disease symptoms.

Nanoparticles fabricated from Beta 1, 3-D-glucan (from bakers' yeast), GeRPs, encapsulating *Map4k4* siRNA (20 μg/kg) were designed to be taken up by macrophages in the GALT. Following oral gavage, GeRPs inhibit LPS-induced TNF-alpha production in serum and peritoneum (Aouadi et al., 2009).

Amphiphilic polyallylamine-base polymeric micelles formed nanocomplexes (150–300 nm) with siRNA (Guo et al., 2013). The resulting complexes were stable in simulated gastric/intestinal fluids achieving cellular uptake, endosomal release and gene knockdown in a Caco-2 cell model.

Bio-responsive formulations have also been designed to achieve oral delivery of therapeutic NAs. Thioketal NPs were formulated using a novel polymer poly-(1,4-phenyleneacetone dimethylene thioketal)

Table 1
Formulation approaches for in vivo intestinal delivery of therapeutic nucleic acids.

Gene target	Formulation	Therapeutic application/ disease model	Delivery route, dose and response	Reference
siRNA				
TNF-alpha	Lipofectamine 2000	Mouse colitis	Rectal delivery. Gene silencing in colon, reduced histopathological disease	(Zhang et al., 2006)
Cyclin-D1	Lipid vesicles labelled with hyaluronan; Antibodies to Beta7 integrin	Mouse colitis	Intravenous (2.5 mg/kg). Reduced gut inflammation, reduction in expression of TNF-α and IL-12	(Peer et al., 2008)
Map4k4 (TNF-alpha)	GeRPs targeting GALT	C57BL6/J male mice	Oral (20μg/kg); attenuated *Map4k4* mRNA expression in PECs and macrophages from spleen, lung and liver	(Aouadi et al., 2009)
TNF-alpha	Thioketal bioresponsive (ROS) polymer	Ulcerative colitis	Oral (0.23 mg/kg); reduce the colonic mRNA levels of proinflammatory cytokines	(Wilson et al., 2010)
TNF-alpha	siRNA.PEI complex loaded into PLA NPs	LPS treated mice	Oral; TNFa siRNA/PEI-loaded NPs induced a significant decrease in TNFa secretion (175.9 pg/mL vs 559.8 pg/mL)	(Laroui et al., 2011)
TNF-alpha/cyclin D1	NiMOS	IBD Mouse	Oral (1.2 mg/kg); Synergistic effect with dual siRNAs	(Kriegel and Amiji, 2011)
TNF-alpha	Amphiphilic cationic CDex NP	IBD mouse	Rectal delivery, reductions in total colon weight and colonic mRNA expression of TNF-α (73%) and IL-6 (58%) vs controls	(McCarthy et al., 2013)
TNF-alpha	P(CBA-bPEI)-PEG-Mannose	DDS colitis	Ex vivo, colitis tissue from DDS mouse; 60% reduction in TNF-alpha expression vs control	(Xiao et al., 2013)
Map4k4	Galactosylated trimethyl chitosan-cysteine NPs	DDS colitis	Oral 250 μg/kg daily for 6 days; significantly improved DSS-induced body weight loss, colon length shortening, and increase of myeloperoxidase activity	(Zhang et al., 2013)
TNF-alpha	Chitosan NPs	C57BL/6J mice	Oral gavage mice (28 μg); biodistribution detected NP in colon 1 hr post dosing	(Ballarín-González et al., 2013)
Klf4	PLA	DSS colitis	Oral delivery (0.5 ng daily); reduced clinical symptoms & MPO activity	(Ghaleb et al., 2014)
TNF-alpha	Fab labelled PLGA-PEG NP	DDS colitis mouse	Oral delivery (60 μg/kg); Fab′-bearing NPs loaded with TNFα siRNA attenuated all parameters of colonic inflammation (e.g., weight loss, myeloperoxidase activity, and Ικbα accumulation) vs the untargeted NP.	(Laroui et al., 2014)
CD98	UAC (polymer)-PEG NPs labelled with Ab to CD98in a chitosan alginate hydrogel	Chronic (RAGI-/- mice; acute colitis DDS mice	Oral delivery (1-2 mg/kg); deduced expression of CD98, TNF-a%IL-12	(Xiao et al., 2014)
Survivan shRNA-expression pDNA & VEGF siRNA	Galactose modified trimethyl chitosan-cysteine conjugate	SC tumour mouse model	Oral delivery (20μg pDNA&4μg siRNA); synergistic effect with the dual treatment; silencing survivan & VEGF &tumour regression	(Han et al., 2014)
TNF-alpha	Mannose-modified trimethyl Chitosan-cysteine (MTC)	Sprague-Dawley rats	Oral (50μg/kg) inhibited TNF-a expression in macrophage enriched liver, spleen & lung tissue	(He et al., 2015)
TNF-alpha, IP-10/KC	CaP/PLGA/PEI	DSS colitis mouse	Rectal admin (12μg NP) reduced expression of TNF-a (40%) & IP-10 (50%) in colon	(Frede et al., 2016)
CD98	Ginger-derived lipid vehicles	FVB mice	Oral; silencing of CD98 in colonic tissue ex vivo	(Zhang et al., 2017)
Akt2	Gold-siRNA-glycol chitosan-taurocholic acid NPs	Orthotopic Colorectal liver metastases mouse model	Oral (25 or 100μg/kg); targeted NPs achieved 43–58% reduction in tumour nodules	(Kang et al., 2017)
pDNA				
Apc	Lipofectamine	Cancer. ApcMin mice	Oral (20 μg DNA). Enhanced gene expression and reduction in polyps	(Lew et al., 2002)
mEPO	Chitosan NP	ICR mice	Oral (50μg DNA). Rapid increase in haematocrit (61%) sustained for 1 week	(Chen et al., 2004)
HBsAg	PLGA microparticles	Mouse, DNA vaccine HBV (Hepatitis B)	Oral (200μg DNA)- gastric juice pre-neutralized. HBsAg expression in GALT; antigen-specific antibody response systemically and mucosally	(He et al., 2005)
IL-10	NiMOS	Colitis mouse	Oral, increased gene expression in colon reversal of disease	(Bhavsar and Amiji, 2007)
IL-22	DOTAP + cholesterol +enhancer	Colitis mouse	Local microinjection into colon (20μg DNA). Reduced inflammation and enhanced mucus production	(Sugimoto et al., 2008)
Transforming growth factor-beta (TGF-β)	Chitosan NP	Ovalbumin-induced allergic BALB/c mice	Oral (50μg DNA). Enhanced TGF-β expression in small intestine; increased OVA-IgA; decreased serum OVA-IgE; reduced histamine release by mast cells into GIT	(Li et al., 2009)

(*continued on next page*)

Table 1 (*continued*)

Gene target	Formulation	Therapeutic application/ disease model	Delivery route, dose and response	Reference
Ag85a (Mycobacterium antigen)	Lipofectamine	Mouse, DNA vaccine vs tuberculosis (TB)	Oral (100μg DNA)- gastric juice pre-neutralized. Induce antigen specific mucosal cellular and humoral immune responses in intestine	(Wang et al., 2010)
GLP-1	PEI NP coated with heparin-taurocholic acid	T2DM Zucker diabetic fatty rat model	normal blood glucose levels were sustained with target NP	(Nurunnabi et al., 2017)
Antisense Oligo				
Rlalpha subunit of protein kinase A	NA	SCID &/nude mice with subcutaneous tumors	Oral. Inhibited tumour growth, prolonged survival time	(Wang et al., 1999; Tortora et al., 2000)
TNF-alpha, ISIS 2503 & 104838	Sodium caprate	Intra-intestinal catheterised pig model	Intrajejunal (10 mg/kg), bioavailability 2.75% ISIS 104838 2-O-(2-methoxyethyl) modified ASO	(Raoof et al., 2002)
TNF-alpha, ISIS 104838 phosphorothioate antisense oligonucleotide targeting human tumour necrosis factor alpha (TNF-α) mRNA	Enteric coated tablet with sodium caprate	Dogs	Oral (24.93 mg daily for 7 days) bioavailability 1.4%	(Raoof et al., 2004)
TNF-alpha ISIS 104838	Immediate and delayed release minitabs containing sodium caprate	Man	Oral (500–700 mg); 7–12.9% bioavailability	(Tillman et al., 2008)
TNF-alpha	Galactosylated chitosan	Colitis mouse	Rectal delivery. Silencing of TNF-Alpha, reduced disease severity	(Zuo et al., 2010)
TNF-alpha,	Sodium caprate, 2′-O-methyl phosphorothioate ASO	Muscular dystrophy, mdx-mouse	Intestinal infusion (250–1000 mg/kg); lower plasma levels vs IP and SC enhanced with sodium caprate	(van Putten et al., 2014)

(PPADT) that degrades selectively in response to elevated reactive oxygen species (ROS) at inflamed sites in IBD (Wilson et al., 2010). Oral administration of the TNF-alpha siRNA loaded NPs to a mouse UC model resulted in localised delivery to the colon, reduced cytokine levels resulting from gene silencing and alleviation of the disease partly due to the enhanced stability of the NPs in the GI tract.

A TNF-alpha siRNA loaded nanogel encapsulated in a P(MAA-co-NVP) polymer protected the nanogel from release in the gastric acidic pH. At the higher intestinal pH of 6–7.5 the polymer swells facilitating enzymatic degradation of the polymer matrix and release of the nanogel for cellular uptake. The enzyme and pH-responsive formulation achieved cellular uptake in murine macrophages resulting in knock-down of TNF-alpha (Knipe et al., 2016).

3.2.2. Colon cancer

Another obvious disease target for oral delivery of nucleic acid-based therapeutics is colon cancer. Here again local delivery and uptake into the tumour is desirable and active targeting using a receptor ligand approach is an attractive possibility. A variety of tumour targeting ligands has been identified (Riaz et al., 2018) and specific examples including a 12-residue peptide that interacts with integrin alpha6beta1 over-expressed on colon cancer cells has shown promise (Ren et al., 2016). Mutations of the adenomatous polyposis coli (APC) gene are frequently associated with the development of colorectal cancer. Gene replacement therapy has shown promise in animal models of the disease. Following oral administration of a lipofectamine-based liposomal formulation containing a functional APC gene to APCmin mice a 54% reduction in intestinal polps was detected (Lew et al., 2002). Nanoparticles formulated with galactose modified trimethyl chitosan-cysteine conjugate were used to co-deliver survivan shRNA-expression pDNA and vascular endothelial growth factor siRNA via oral administration (Han et al., 2014). The tumour animal model was established following subcutaneous injection of hepatoma H-22 ascites or BEL-7402 cells. Following oral administration, the NPs penetrated the gut wall, attributed to mucoadhesion and opening of TJs, and accumulated in the tumour tissue. The co-delivery of shRNA and siRNA (daily for 20 days, 1 mg pDNA/kg and 200 μg siRNA/kg) mediated a synergistic therapeutic response, resulting in silencing the expression of both survivan and VEGF and significant tumour regression which varied with ligand grafting density.

The same group (Yin and colleagues) synthesized a modified chitosan with histidine and cysteine amino acids (Zheng et al., 2015). This vector was used to deliver survivan shRNA in a SC xenograft hepatoma tumour mouse model. The vector was designed to overcome the barriers to delivery at the cellular level including, cellular uptake, endosomal escape (histidine), increased nuclear localisation and glutathione mediated release (cysteine). Following oral administration (20 μg shSur-pDNA per mouse per day for 18 days), tumour growth was retarded and surviving was extended due to increased apoptosis.

Oral delivery of siRNA has also been investigated to treat colorectal liver metastases (CLM) formed from colorectal cancer (Kang et al., 2017). This study describes the formulation of gold (Au). siRNA nanoparticles coated with glycol chitosan and targeted with taurocholic acid (TCA). The TCA was selected to protect the siRNA from gastrointestinal degradation and ensure targeting to the CLM via enterohepatic circulation. The Akt siRNA initiates apoptosis and the efficacy was evaluated by monitoring expression of the apoptosis markers; Bax, caspase-9 and PARP. Detailed mechanistic studies illustrated vesicular mediated (receptor mediated transcytosis) uptake of the TCA targeted nanoparticles from the ileum, transport from the ileum to the liver was confirmed by identification of the nanoparticles in the hepatocytes. The presence of the nanoparticles in the kidneys suggests elimination by the kidneys. Following oral administration (siRNA 25 or 100 μg/kg) using an orthotopic CLM animal model (established by injection of CT26 cells into the spleen), the therapeutic efficacy of the targeted nanoparticles was significantly greater versus the untargeted Au-chitosan nanoparticle. The TCA targeted nanoparticles produced a 43–58% greater reduction in the

number of tumour nodules via induction of cell apoptosis by regulating the Akt/PI3K pathway.

Enterohepatic recycling via the bile acid pathway has also been exploited for oral delivery of GLP-1 pDNA complexed with PEI and coated with heparin-taurocholic acid (Nurunnabi et al., 2017). Therapeutic efficacy was confirmed in a genetically engineered T2DM Zucker diabetic fatty rat model where normal blood glucose level was sustained following oral administration of the TCA targeted GLP-1 nanoparticle.

3.2.3. Microbiome

One approach to trigger the release in the colon which has attracted recent attention is based on exploiting the microbiome. There has been an explosion of interest detailing the role of the microbiome as a determinant of the health status of the human host and in the development of new biotherapeutics (Gibson et al., 2017; Prakash et al., 2011; Serban, 2014). Recent studies have illustrated that the gut microbiota in IBD patients is altered versus healthy gut (Nishida et al., 2018; Sheehan and Shanahan, 2017). This provides opportunities to exploit 'microbiome triggered' site specific drug delivery by utilizing materials, mainly polysaccharides, selectively metabolized by the unique IBD microbiota (Jain et al., 2014; McConnell et al., 2008). Matching the delivery technology to the microbiota may enable targeting of nucleic acids to the colon for treatment of diseases including IBD and cancer.

3.3. ADMET

3.3.1. Pharmacokinetics and biodistribution

Typically following IV administration siRNA it is rapidly eliminated and displays a short half-life ranging from seconds to minutes (Evans et al., 2016; Godinho et al., 2014; O'Neill et al., 2013). Circulation time can be extended by complex formation with for example cationic vectors and by PEGylation (Kolate et al., 2014; Miteva et al., 2015). The reasons for the short half life of siRNA and some of the cationic formulations include; enzymatic degradation by RNase, interaction with serum proteins causing siRNA release or nanoparticle aggregation and opsonisation (Park et al., 2016). For oral administration some of these issues, such as interactions with plasma proteins and the toxicity of cationic materials, may not be problematic. In contrast, other barriers arise such as the need to resolve passage through the stomach, degradation by the digestive enzymes, mucus and poor membrane permeability, as discussed in Section 2 above.

Limited PK data has been published following oral administration of nucleic acids. Han et al. (2014) monitored distribution into plasma following oral administration of 20μg FTTC-pDNA and 4μg TAMRA-siRNA formulated in a multifunctionalised chitosan-based vector. The nanoparticles increased the percentage distribution in plasma by approximately 7-fold relative to naked siRNA with maximum amounts detected at around 6 hour post administration, similar results were reported with the pDNA. Simultaneously, the percentage distribution in the intestine decreased rapidly over the first 6 h while values in the tumour increased reaching a maximum at around 11 hour post administration.

Direct northern and quantitative polymerase chain reaction (PCR) were used to detect the disposition of siRNA.chitosan nanoparticles (Ballarín-González et al., 2013). In contrast to oral dosing of naked siRNA (78 μg per mouse) the chitosan nanoparticles maintained the stability of the siRNA and were detected in the stomach, proximal and distal small intestine, and in the colon as early as 1-hour post dosing and persisted for up to 5 h. Biodistribution studies, 1-hour post dosing, also indicated intact siRNA in the liver, spleen and kidney although the levels varied depending on the N:P ratio of the nanoparticles.

Trimethyl chitosan cysteine nanoparticles containing TNA-alpha siRNA were modified by mannose to target macrophages following oral delivery (50 μg/kg) (He et al., 2015). Absorption occurred via enterocytes and Peyers patches resulting in reduced systemic production (serum) of TNF-alpha. Distribution to macrophage-enriched reticuloendothelial tissues including liver, spleen and lung resulted in decreased tissue levels of TNF-alpha mRNA in an acute hepatic injury rat model thus protecting the animals from further hepatic damage.

Kang et al. (2017) also looked at the biodistribution of the gold (Au). siRNA nanoparticles coated with glycol chitosan and targeted with taurocholic acid (TCA) for the treatment of colorectal liver metastases (CLM). Using a fluorescent marker, results indicated that, relative to the untargeted nanoparticles, the levels of the targeted formulation were 1.2 to 1.4-fold greater in the ileal and liver at 6 and 12 h respectively. A more detailed biodistribution study over 48 h confirmed the higher levels in the liver with the TCA targeted nanoparticles indicating the successful exploitation of the enterohepatic circulation mechanism for targeting to the CLM.

Uptake by the bile acid transporter in the ileum and transport to the liver following oral administration of taurocholic acid targeted nanoparticle (HTCA) containing GLP-1 pDNA was also confirmed by biodistribution studies (Nurunnabi et al., 2017). High levels of the HTCA nanoparticles were detected in the ileum and liver 3 hour post oral administration. Comparative distribution studies indicated that the nontargeted formulations remained stuck in the GIT, in contrast the HTCA nanoparticles survived the gut environment were absorbed from the ileum and were detected in the liver.

Using a lipid-based NP (LNP) containing siRNA Ball et al. (2018) investigated the distribution of fluorescently labelled naked siRNA and LNPs in the intestine over 8 h after oral administration. By 30 min both the naked siRNA and the LNP had travelled through the stomach and arrived in the small intestine. Over 8 h the fluorescent signal in the colon increased while the overall signal decreased with time indicating elimination from the gut. No fluorescence was detected in the kidneys, heart, liver, spleen or pancreas suggesting that neither the siRNA nor the LNPs crossed the intestinal membrane into the blood. Although the LNPs arrived in the intestine no gene silencing was detected after 24 h in the gut samples taken possibly due to an insufficient dose (5 mg/kg siGAPDH).

In summary, the in vivo studies reviewed above show that orally administered nanoparticles containing nucleic acid distribute to various locations along the GIT including the colon and achieve cellular uptake thus facilitating local therapeutic effects. Formulations may need to be modified to ensure maximum retention locally in the GIT to avoid unwanted systemic effects. In some cases, systemic absorption has been reported with distribution mainly to the liver this is useful where treatment of liver disease is required.

4. Pre-clinical models

A wide range of models have been developed over the years to assess intestinal delivery mainly with traditional low molecular weight drugs in mind. Many of these models can equally be applied to assess intestinal delivery of macromolecules (Harloff-Helleberg et al., 2017). While models simulating the healthy gut can provide useful information the physiological environment may be altered significantly by disease (e.g. IBD, cancer) and this should be taken into consideration as the resulting changes will likely impact on the performance of the delivery system (Hua et al., 2015). Data using such models have previously been discussed in Section 3 above. The physiological barriers to non-viral intestinal delivery of gene therapeutics and the pre-clinical models used to assess delivery in disease conditions has previously been reviewed (O'Neill et al., 2011b) consequently this review will focus on more recent developments.

4.1. In vitro models

4.1.1. Models of the extracellular GIT environment

A range of biorelevant media have been developed to reflect the varying luminal conditions of the GIT in the fed and fasted states. These media reflect differences in pH, buffer capacity, the presence of bile salt and enzymes in the various parts of the GIT, stomach, small and large intestine (Markopoulos et al., 2015; Singh et al., 2015). While such media were initially designed to assess drug dissolution, they have been

utilized to quantify the intestinal stability of nucleic acids in non-viral delivery formulations (Guo et al., 2013; McCarthy et al., 2013; O'Neill et al., 2013). More recently biorelevant media designed to reflect intestinal disease states have been proposed (Effinger et al., 2018b). Changes in hydrodynamics, luminal contents and microbiota in a healthy gut were compared to those reported for patients suffering from ulcerative colitis, Crohn's disease, coeliac disease, irritable bowel and short bowel syndromes. This bank of information will inform the design of disease-specific biorelevant media which could be used to study gene therapeutics engineered to target particular intestinal diseases (Effinger et al., 2018a, 2018c).

The presence of a mucus layer on the surface of the intestinal membrane may represent a diffusional barrier to gene therapy (Duncan et al., 2016) and various technological strategies have been explored to overcome this barrier (as discussed above in section 3.1.2.) (das Neves and Sarmento, 2018). A wide variety of methods to investigate the diffusion of drugs and particles in mucus has recently been reviewed including the use of in vitro isolated mucus models (Lock et al., 2018). The source of mucus (native versus commercial), the collection, preparation, characterization and storage of samples may influence results produced and consequently all need to be carefully controlled. Experimental techniques used to explore the barrier functions of mucus include multiple particle tracking (MPT) (Lai et al., 2007), fluorescent recovery after photobleaching (FRAP) (Yildiz et al., 2015), penetration studies and bulk diffusional studies (Groo et al., 2013). While valuable information can be obtained via techniques such as MPT and FRAP specialised equipment and trained personnel are required and therefore they may not be conducive to routine screening. In contrast to MPT which facilitates the study of single particle dynamics, simpler techniques utilizing capillary tubes (Popov et al., 2016) or membranes are used to investigate bulk particle transport (Groo et al., 2013; Groo et al., 2014).

4.1.2. Cell culture models

A range of intestinal cell culture models have been used to assess intestinal delivery of gene therapeutics (O'Neill et al., 2011b). The CaCo-2 model has been used to quantify the extent of cellular uptake, the degree of transfection and intracellular trafficking of non-viral gene delivery vectors (Cryan and O'Driscoll, 2003; O'Neill et al., 2011a). The influence of mucus, which may be altered in disease conditions thus influencing uptake, as a diffusional barrier to gene therapeutics has been investigated using co-culture models of Caco-2 and mucus secreting cells such as Ht29GlucH (Cryan and O'Driscoll, 2003; O'Neill et al., 2013). If particulate uptake via M-cells is the aim, models incorporating M-Cells in an intestinal follicle-associated epithelium (FAE) may be useful (Wilson et al., 2010). M cells significantly influenced the uptake and localisation of pDNA-loaded chitosan and trimethylchitosan nanoparticles (Plapied et al., 2010).

Cell models representative of the disease of interest may provide more useful information (Guo et al., 2016). In the case of IBD a variety of cell models incorporating macrophages such as RAW264.7 has been used, which when stimulated with LPS secrete elevated levels of cytokines characteristic of IBD (McCarthy et al., 2013). The formation of a co-culture incorporating macrophages, Caco-2, Ht29 or FAE cells to form a more physiologically relevant model may also be possible (O'Neill et al., 2011b).

More recently interest has grown in the potential to create 3D cell-based models to help simulate more accurately the in vivo environment (Fitzgerald et al., 2015). It has been shown that cells grown in a 3D architecture behave differently to those grown in 2D likely due to the intimate cell-cell contact and the resulting cross-talk. Three dimensional models of the intestine using collagen (Yu et al., 2012) and hydrogel (Dosh et al., 2017) scaffolds have produced drug permeability values reflective of in vivo data. To replicate the topography of the intestine cross-linked collagen hydrogel was molded into crypt and villi-like structures. Human small intestinal cells were shown to proliferate on these scaffolds and underwent differentiation in response to chemical stimuli thus creating a model which replicates the anatomical shape of the human small intestine (Wang et al., 2017). Using a triple co-culture model of intestinal myofibroblasts (CCD18-Co cells) embedded in Matrigel, onto which epithelial enterocytes (Caco-2 cells) and mucus-producing cells (HT29-MTX cells) were seeded, Pereira et al. (2015), showed that the CCD18-Co cells secreted fibronectin which acted as a type of ECM supporting the cells in a 3D architecture. This mucus secreting co-culture model was successfully used to evaluate intestinal permeability of insulin.

Recent microfluidic human intestine chip models have been described where human intestinal cells, capillary endothelium, immune cells and commensal gut microbiome grow, coexist and interact under conditions of constant flow representative of peristalsis (Bein et al., 2018). While early studies were done using Caco-2 cells, a primary human small intestine-on-a-chip has been established using human enteroids from patient duodenal biopsies (Kasendra et al., 2018). The epithelial cells were grown on a porous membrane within the device with human intestinal microvascular endothelius cultured in a parallel channel. The use of primary cells provides the opportunity to use the patients own cells and allow personalised therapies to be developed. Human gut-on-a-chip microfluidic devices have been used to study intestinal pathophysiology and mechanisms of disease. For example, such a device was used to investigate the interplay of the gut microbiome and suppression of peristalsis on inflammation like that seen with IBD (Kim et al., 2016), and similar devices have been suggested to investigate colorectal cancer (Pereira et al., 2016).

This chip microfluidic technology has also been used to establish a multi-organ arrangement allowing cross talk between different tissues. One multi-organ model, comprised of liver cells (hepatocytes and Kupffer cells) integrated with intestinal cells (enterocytes, goblet cells and dendritic cells). Using a range of biomarkers, gut-liver cross-talk which is essential for normal physiology was established and the model was used to study liver-gut inflammatory interactions (W.L.K. Chen et al., 2017), and to investigate pharmacokinetic parameters including intestinal drug permeability and hepatic metabolism relevant following oral administration (Tsamandouras et al., 2017).

The microfluidic-chip technology, particularly the intestinal models with capacity to reflect disease conditions, maybe a very powerful in vitro tool to assess intestinal gene therapeutics.

4.2. Animal models

A variety of animal models including ex vivo, where tissues are removed from the animal, in situ, where the animal has been surgically manipulated and in vivo animal models, both healthy and diseased, have been used to evaluate intestinal delivery of gene therapeutics (Cryan and O'Driscoll, 2003; Guo et al., 2016; McCarthy et al., 2013; O'Neill et al., 2011a). The majority of these studies have been performed in rodents (mice and rats). A recent review on the ability of the pig model to predict oral bioavailability in man (Henze et al., 2019) has shown that while similarities in terms of GIT anatomy and physiology exist species differences in individual drug metabolism have also been suggested. The correlation between human and pig bioavailability, for a limited bank of drugs, was comparable to that achieved between human and dog. Consequently, the pig model may be a worthwhile model for future studies with nucleic acid therapeutics.

4.3. Disease models

IBD are very complex diseases (Walsh et al., 2013) and it is difficult to get a pre-clinical model which truly represents the complexity. Over 50 experimental colitis models (mice and rats) have been described, they can be generally classified as; chemically induced, resulting from genetic manipulation or those dependent on transfer of cells from donors to immunodeficient recipients (Jiminez et al., 2015; Kiesler et al.,

2015; O'Neill et al., 2011a). In the studies reviewed above (Section 3.2), the most common model used involves induction of inflammation by chemical means, e.g. by administration of dextran sodium sulphate (DSS) in the drinking water which leads to disruption of the epithelium. Other agents used include rectal administration of trinitrobenzene sulfonic acid, which renders colonic proteins immunogenic to the host immune system thereby driving a mucosal immune response that initiates colitis (Kiesler et al., 2015). Administration of LPD has also been used to stimulate development of inflammation and this model was used by Aouadi et al. (2009) to assess the therapeutic value of GeRPs containing siRNA following oral administration. Genetically manipulated animals have also been used including the IL-10 knockout mouse (Kühn et al., 1993). These animals develop inflammation of the colon characterized by the existence of an inflammatory infiltrate composed of lymphocytes, macrophages and neutrophils. Finally, adaptive transfer colitis, involves the transfer of naïve CD4+ T cells (CD4 + CD45RBhigh T cells) from donor mice to immunodeficient SCID or Rag1 −/− recipient mice. This model was used by Xiao et al. (2014), to investigate the efficacy of an antibody targeted NP loaded with siRNA to alleviate colitis.

To achieve maximum efficacy from nucleic acid-based therapeutics it is essential to identify a suitable gene target which can be a challenge given the complex pathology of the disease. In the studies reviewed above (Section 3.2) the most common targets for gene silencing were TNF-alpha, IL-6, cyclin D1 and Map4k4 (Guo et al., 2016; Walsh et al., 2013). The success of treatments is frequently assessed by monitoring clinical signs of the disease such as changes in body weight, stool consistency and rectal bleeding. In addition, markers of the disease including colon length and weight, markers of inflammation for example proinflammatory cytokines, colon histology and myeloperoxidase activity have also been utilized (McCarthy et al., 2013; O'Neill et al., 2011a).

Colorectal cancer (CRC) a variety of models has been described (DE-Souza and Costa-Casagrande, 2018; Johnson and Fleet, 2013) but most can be classified as chemically/environmentally induced, cancers caused by genetic manipulation, or by injecting cancer cells locally including subcutaneous xenografts or systemically to produce orthotopic tumors (Han et al., 2014; Kang et al., 2017). The chemical carcinogenic agents used include 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM) which is an active metabolite of DMH. Both are reported to initiate tumors by a mechanism similar to that which occurs naturally in humans (Perše and Cerar, 2011).

Due to the increasing level of knowledge re the genetic basis of CRC numerous genetically modified animal models exist. The most commonly used models are based on mutations in the APC gene, p53 and K-ras, the advantages and disadvantages of these models has previously been reviewed (DE-Souza and Costa-Casagrande, 2018).

4.4. In silico

Physiologically based pharmacokinetic (PBPK) models utilise a blend of in vitro pre-clinical data, including the physicochemical properties of drugs, together with physiological conditions to predict in vivo bioavailability for example following oral drug administration (Kostewicz et al., 2014). There are several software packages commercially available including GastroPlus 8.0, Simcyp 13.3 and GI-Sim 4.1, generally these are used to predict absorption of small molecular weight drugs (Sjögren et al., 2016). Application of these modelling packages to macromolecules such as nucleic acids is challenging due to the physicochemical properties and the potential for degradation in the lumen and in the gut wall. In addition, basic data used to produce predict performance in man including permeability data is not readily available. With the exception of a study on patients following bariatric surgery (Darwich et al., 2012; Darwich et al., 2013; Sjögren et al., 2016) no other PBPK model exists for patients with GI disease. Potential exist to combine data generated using disease-specific biorelevant media with PBPK models to evaluate the performance of orally administered gene therapeutics (Effinger et al., 2018a; Otsuka et al., 2013).

All animal models have advantages and disadvantages. It is important particularly in disease-based models to understand the limitations of the model in terms of replicating the human condition. The choice of a suitable model can often depend on the aims of the study and this is worth considering when designing the experimental protocol. The field of oral administration of nucleic acid therapeutics is still in its infancy and more work is needed to establish in vitro in vivo correlations and to help build confidence in particular models to accurately predict responses in man.

5. Outstanding barriers to translation

A series of outstanding barriers need to be overcome for the translation of nucleic acid-based therapeutics to oral drug products. The first barrier is related to chemistry, manufacturing and controls (CMC) of the advanced drug delivery systems to comply with the current good manufacturing practices (cGMP) (Jeevanandam et al., 2016; Tyagi and Santos, 2018) and the cost associated with the development and manufacturing of these systems. The second barrier is linked with the clinical challenges due to the poor translation of positive results observed in preclinical models into human (Jeong et al., 2016; Tyagi and Santos, 2018) and the specific health authorities' questions on pharmacokinetics (PK), pharmacodynamics (PD) and in vivo targeting efficiency to avoid off-target effects (Acharya et al., 2017).

5.1. Chemistry, manufacturing and control

The advanced drug delivery systems (DDS) needed to unlock the therapeutic efficiency of nucleic acid-based drugs should comply with the cGMP and ICH quality guidelines (e.g. ICH Q8(R2), (International Conference of Harmonisation, 2009)) as any drug products. These guidelines emphasize the need to identify the critical quality attributes (CQA) of the drug product in relation with its biological activity. This is needed to ascertain quality, safety and efficacy of drug products and ensure batch-to-batch reproducibility.

The chemistry of materials used in the advanced DDS and their critical material attributes (CMA) on the drug product properties should be identified. For materials possessing a compendial monograph and precedence of use in marketed drug product (e.g. Inactive Ingredient Database (IID) listing, (U.S. Food and Drug Administration, 2018)), the filing process is easier than for new materials. Most of the materials used in the preparation of the advanced DDS described in this manuscript: SEDDS, liposomes and nanoparticles are already listed in Pharmacopeial monographs and used in drug products by either oral route or injection. The exception would be for transfection agent like Lipofectin or cationic polymers like polyethyleneimine or thiolated chitosans that do not have yet compendial monograph or precedence of use in drug products. These new materials are essential for the performance of nucleic acid-based therapeutics but will be considered as new chemical entities by national authorities and the translation of these molecules to the market will be very costly as safety of these materials should be investigated as for any new molecules.

The manufacturing process used for the preparation of advanced DDS should be described and critical process parameters (CPP) should be identified to ascertain the control of the manufacturing process at different scales and overtime. For SEDDS the manufacturing process is quite simple as it consists of the mixing of materials to obtain an isotropic solution of drug substance, surfactants, oils and solvent and then the encapsulation of the solution in capsules. This process is already widely used by the Pharmaceutical industry to manufacture SEDDS of poorly water-soluble drugs as well as peptides (e.g. Cyclosporine A). A market research analysis with PharmaCircle performed on April 19th, 2018 has allowed the identification of 150 marketed oral drug products

worldwide with the drug delivery keyword 'Lipid & SEDDS'. A vast majority of these products are (or were) marketed in highly regulated countries (108 are marketed in the USA for example) showing that these formulations classically encapsulated in soft gelatin capsules (73%) or hard capsules (23%) do comply to cGMP. The excipients' composition of 86 drug products was available in the database to classify these formulations according to the Lipid Formulation Classification System (LFCS, (Pouton, 2006)). One quarter of these formulations can clearly be identified as SEDDS – also referred as LFCS Type II or Type III – with the use of self-emulsifying excipients, and the rest of these formulations are mostly lipid solutions - LFCS Type I. The fact that SEDDS are already marketed worldwide in oral drug products is a major advantage for the seamless clinical translation of nucleic acid-based therapeutics at a minimal cost. In addition, these capsule formulations are generally stable in standard conditions for at least three years.

Manufacturing process and scale-up of nano DDS like liposomes and nanoparticles on FDA cGMPs in large scale production are more difficult and costlier (Jeevanandam et al., 2016) than classic tablet or capsule manufacturing processes. This may be a reason why the PharmaCircle market analysis with the keyword 'Liposome' for oral marketed drug products has reported only one reference. This is a product named SiderAl Forte marketed in Italy by BMG Pharma S.r.l. However, several marketed references of liposomes are available by injection and prove that the manufacturing and scale-up of this type of formulation is achievable even in sterile conditions.

Finally, the market analysis with the keywords 'Nanoparticles', 'Polymeric nanoparticles', 'Lipid nanoparticles' has resulted with no marketed oral drug product. Lipid nanoparticle (LNP, Cullis and Hope, 2017) is scalable as one product has recently been approved by the US FDA: LNP TTR-siRNA drug (Patisiran). Alike for liposome, lipid nanoparticles are already used in nucleic acid-based nanotherapeutics by injection. These LNPs are stable at least one year at 4 °C. Overall, one advantage for oral delivery of nucleic acid-based therapeutics versus injection is that the sterilization step is not needed and would decrease significantly the cost of goods.

The physicochemical characterization of advanced DDS is needed to link the biological activity of the nucleic acid-based therapeutics with the properties of the formulation. The nanoformulations are characterized by their size distribution (mean, polydispersity index), morphology and texture, their charge, surface chemistry and ability to retain the drug substance in the nanocarrier along the GIT. In the case of liposomes and nanoparticles, these properties are achieved after the manufacturing process and should be characterized to ascertain the control of the manufacturing process. However, for SEDDS that are isotropic solutions of drug substance and excipients the nanodroplets or micelles are only formed in situ after the ingestion of the formulation. Appropriate and biorelevant characterization methods should be implemented to verify the self-emulsification of the formulation and the obtention of specific phases that allow maintaining the drug in colloids (Jannin et al., 2015).

5.2. Clinical challenges and associated regulatory hurdles

No clinical trial for oral dosing of siRNA and pDNA were reported on clinicaltrials.gov on January 1st, 2019. However, many clinical studies where the nucleic acid-based therapeutics are dosed by injection or surgery are reported. Within the 239 studies listed for pDNA and 60 for siRNA, some advanced DDS described in this manuscript for oral dosing were tested in clinical trials by injection. Lipid nanoparticles were the most common DDS used by intravenous administration for the delivery of siRNA.

The clinical challenge of nucleic acid-based therapeutics is due to the inability of most in vitro models and preclinical models to mimic human with specific disease conditions (see Sections 4.1 and 4.2).

The next barrier to clinical translation is the PK, PD, toxicology and biocompatibility of nucleic acid-based therapeutics (Acharya et al., 2017). Nucleic acid-based therapeutics possess inherent poor pharmacokinetic properties (short biological half-life, poor penetration, and non-specific stimulation of the immune system) and need to be delivered orally with a potent targeted DDS. Hence the final major regulatory hurdle for clinical translation is the in vivo targeting efficiency of the DDS (Jeong et al., 2016) in order to avoid off-target silencing for example with siRNA (Acharya et al., 2017). Advanced DDS such as LNP are of prime importance (Granot and Peer, 2017) to allow accumulating nucleic acid-based therapeutics in targeted organs and thus inducing a therapeutic effect at a dose with an economically acceptable cost. These DDS should minimize the suppression of genes other than the targeted genes and the immune stimulation.

Finally, the approval of Patisiran - the first nucleic acid-based therapeutics will pave the way for further and streamlined drug development. It will give to the industry a clear roadmap of what FDA requires in clinical development to ascertain the control of the targeted nucleic acid-based therapeutics.

6. Conclusions and future prospects

Nucleic acids administered by the oral route must overcome physicochemical and physiological barriers. In particular, nucleases present in the intestinal lumen and in intestinal cells cause the degradation of RNA and pDNA. Their high molecular weight and high negative charge make the crossing of the mucus layer and cell membrane difficult. Therefore, formulation strategies have been developed to overcome this enzymatic barrier and to enhance the nucleic acid absorption across the intestinal barrier. Nevertheless, even with these optimized formulations, the overall systemic bioavailability of nucleic acid remains very low. Hence, the oral delivery of nucleic acid is mainly intended for their local administration rather than for their systemic use. The most obvious and most investigated biomedical applications are IBD and to a lesser extend colorectal cancer. Many preclinical studies tend to indicate that local delivery of formulated RNA could be translated to clinical use. However, no clinical studies confirm the potential of orally delivered nucleic acid-based therapeutics.

The future trends for the oral delivery of nucleic acid-based therapeutics will rely on the future developments in molecular biology and new knowledges in disease physiopathology to identify new targets for gene therapy e.g. the identification of new genes involved in IBD. Moreover, better formulations adapted both to protect the nucleic acid from degradation and enhance its cellular permeation as well as to deliver it to the target site in the gastrointestinal tract will be required. Multicomponent systems composed of a nucleic acid delivery system such as nanoparticulate systems and a more conventional part for oral delivery need to be optimized. In particular, based on their easy and controlled formulation and manufacture, their well-characterized pharmaceutical properties and the approval of most excipients by regulatory agencies, SEDDS could potentially be promising for the oral delivery of nucleic acid and require further preclinical and clinical studies.

In conclusion, it is difficult at this stage to provide a clear timeline for clinical translation of oral nucleic acid therapeutics as many formulation and regulatory barriers remain outstanding, however existing pre-clinical data look convincing and particularly promising for local delivery.

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